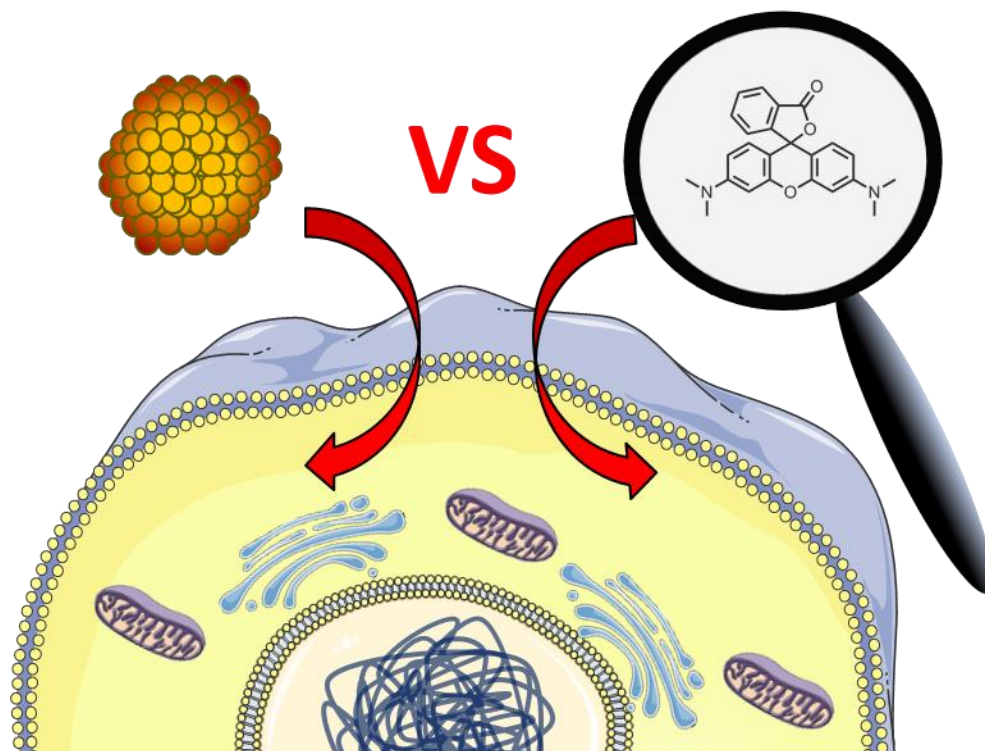


Cellular uptake of nanoparticles versus small molecules: a matter of size

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CONSPECTUS: The primary function of the cell membrane is to protect cells from their surroundings. This entails a strict regulation on controlling the exchange of matter between the cell and its environment. A key factor when considering potential biological applications of a particular chemical structure has to do with its ability to internalize into cells. Molecules that can readily cross cell membranes are frequently needed in biological research and medicine, since most therapeutic entities are designed to modulate intracellular components. However, the design of molecules that do not penetrate cells is also relevant toward, e.g. extracellular contrast agents, which are most widely used in clinical diagnosis.

Small molecules have occupied the forefront of biomedical research until recently, but the past few decades have seen an increasing use of larger chemical structures, such as proteins or

nanoparticles, leading to unprecedented and often unexpectedly novel research. Great achievements have been made toward understanding the rules that govern cellular uptake, which show that cell internalization of molecules is largely affected by their size. For example, macromolecules such as proteins and nucleic acids are usually unable to internalize cells. Intriguingly, in the case of nanoparticles, larger sizes seem to facilitate internalization via endocytic pathways, through which the particles remain trapped in lysosomes and endosomes.

In this Account, we aimed at presenting our personal view of how different chemical structures behave in terms of cell internalization due to their size, ranging from small drugs to large nanoparticles. We first introduce the properties of cell membranes and the main mechanisms involved in cellular uptake. We then discuss the cellular internalization of molecules, distinguishing between those with molecular weights below 1 KDa, and biological macromolecules such as proteins and nucleic acids. In the last section, we review the biological behavior of nanoparticles, with a special emphasis on plasmonic nanoparticles, which feature a high potential in the biomedical field. For each group of chemical structures, we discuss the parameters affecting their cellular internalization, but also strategies that can be applied to achieve the desired intracellular delivery. Particular attention is paid to approaches that allow conditional regulation of the cell internalization process using external triggers, such as activatable cell penetrating peptides, due to the impact that these systems may have in drug delivery and sensing applications. The review ends with a “Conclusions and Outlook” section, where general lessons and future directions toward further advancements are briefly presented.

INTRODUCTION

Cellular uptake is one of the most important processes regulating the biological activity of molecules, and it is determined by the interactions between the molecule and the plasma membrane. The plasma membrane is a thin layer (4-10 nm) that forms the outer boundary of cells. The two main components of this barrier are lipids and proteins; each of them being associated to one of the primary roles of the membrane. Lipids have a morphology task, they form a negatively charged bilayer that constitutes the boundary between the cell and its external microenvironment. On the other hand, proteins have a functional task, they regulate the exchange of substances between internal and external media, and provide cellular signaling.¹

It is vital for cells to tightly regulate the transport of substances through the cell membrane, as exemplified by the fact that around 10% of all human genes are transport related.² Cells use a wide range of transport mechanisms that can be grouped into three classes: simple diffusion, facilitated diffusion, and active transport. *Simple diffusion* is a passive process by which molecules move across the membrane, driven by a concentration or an electric potential gradient, with no need for an intermediary such as a membrane protein.³ This mechanism applies to small and hydrophobic molecules, which can cross directly through the hydrophobic region of the phospholipid bilayer. Conversely, although *facilitated diffusion* is also a passive movement, it occurs with the aid of a membrane protein. Finally, *active transport* requires energy from the cell. Cells use this transport for two reasons: i) to move substances against a concentration or an electrochemical gradient, or ii) to take in larger molecules and particles. When molecules are too large to cross the plasma membrane or to move through a transport protein, cells capture these substances from their outside by engulfing them with the cell membrane itself. The cargos are then separated from the cytoplasm by a lipid membrane, in a process called endocytosis (**Figure 1**).⁴

Endocytosis can be broadly subdivided into phagocytosis and pinocytosis. Phagocytosis is the process by which cells such as macrophages or dendritic cells, engulf particulate matter larger than 0.5 μm and digest it.⁵ On the other hand, pinocytosis is involved in the internalization of fluids and molecules by small vesicles. Pinocytosis can be further subdivided into various categories, depending on the molecular mechanism:

Macropinocytosis: The surrounding fluid is internalized into large vacuoles by actin-dependent membrane protrusion, and results in large intracellular vacuoles ($>0.2 \mu\text{M}$), known as macropinosomes.⁶

Clathrin-dependent endocytosis: A vesicular transport event that cells employ to carry out the endocytosis of activated cell surface receptors.⁷ This occurs by the inward budding of plasma membrane vesicles, containing proteins with receptor sites that are specific to the molecules being internalized.

Caveolin-dependent endocytosis: An endocytic process involving bulb-shaped, 50-60 nm plasma membrane invaginations called caveolae,⁸ whose formation is driven by integral membrane proteins called caveolins.

Clathrin- and caveolin-independent endocytosis: This is a group of several endocytic pathways related to the presence of highly ordered lipid microdomains (40-50 nm in diameter) in the cell membrane, called 'lipid rafts'.⁹

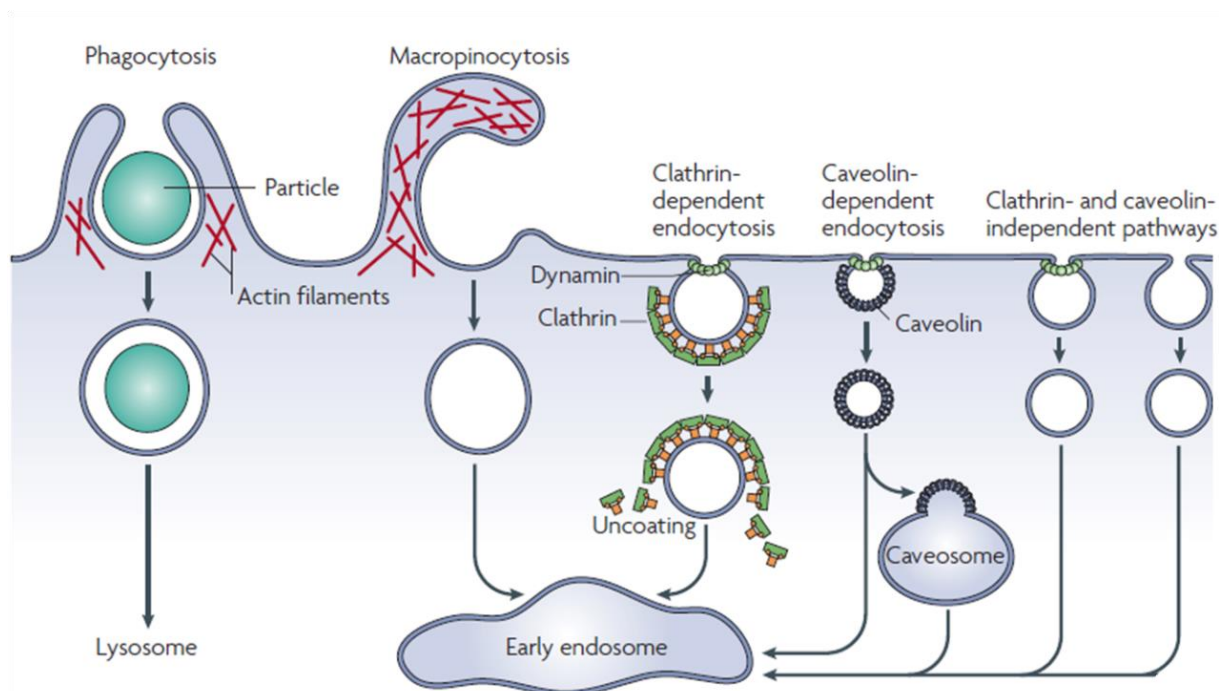


Figure 1. Schematic representation of the endocytic pathways used by cells to internalize molecules. Reproduced with permission from ref. 9. Copyright 2007 Springer Nature.

CELLULAR UPTAKE OF SMALL MOLECULES

We define here small molecules as those with a molecular weight below 1 kDa. It is not by chance that a high percentage of currently used medical drugs fit within this definition. The main reason is that small molecules can be devised to penetrate into cells by simple diffusion, which requires that they are at least slightly soluble in the lipid bilayer.³ Therefore, such small molecules can cross biological barriers, enter cells, and even access cell organelles.¹⁰

One of the best methods to predict the membrane permeability of a molecule is given by Lipinski's "Rule-of-five".¹¹ This rule-of-five is an empirical set of parameters that allow us to predict which compounds are likely to be orally available drugs. However, because bioavailability and

membrane permeability are closely interrelated parameters, the same rule can be used to predict the tendency of molecules to cross the cell membrane by simple diffusion. This rule postulates that poor absorption or permeation is more likely to occur when: i) the molecular weight is above 500 Dalton, ii) the molecule contains more than five hydrogen bond donors, iii) the molecule has more than 10 hydrogen bond acceptors, and iv) the calculated lipophilicity is more than 5.

Another important factor, which is ignored by Lipinski's rule, is the charge of the molecule. Although charged molecules are quite polar, there are several cases in which molecules with one or two positive charges can diffuse through the cell membrane.^{12,13} However, negatively charged molecules usually do not internalize cells efficiently. This effect stems from the fact that the interior of the cell is negatively charged, relative to the extracellular side and, consequently, molecules carrying negative charge would have to move against an electric potential gradient.¹⁴

It is challenging to quantify the cellular uptake of small molecules, with the exception of fluorescent molecules, because the attachment of chemical labels can radically change their biological behavior. Therefore, complex approaches such as radio-labeled molecules or *in vitro* models with synthetic membranes must be used to accomplish the quantification.¹⁵

Improving the cell uptake of small molecules

Prodrug strategy: Lipophilicity is one the main parameters that determine cell uptake by simple diffusion. The lipophilic character of a molecule can be enhanced by means of chemical modifications; the simplest way involves masking polar groups such as carboxylic acids, phosphates, and other charged groups, by forming esters. This strategy is only useful if, after cell internalization, the active molecule can be regenerated by the action of cellular enzymes. Based

on this idea, the approach known as *prodrug strategy* has been developed, which consists of designing an inactive compound containing the parental drug, which undergoes some biotransformation *in vivo* through chemical or enzymatic cleavage, thereby enabling delivery of the active drug.¹⁶ This strategy can be used to improve not only cellular uptake, but also selectivity, and bioavailability. At present, approximately 10% of all drugs used in therapy are administered as prodrugs, and about half of these are hydrolyzed into the active form.¹⁷ One example is cidofovir, a broad-spectrum anti-DNA virus agent, active against herpesviruses. This is a highly polar molecule, which is therefore only slowly taken up by cells through pinocytosis. However, octadecyloxypropyl cidofovir, an alkoxyalkyl ester of cidofovir, can cross the cell membrane by simple diffusion, and the ester gets hydrolyzed by carboxylesterases after internalization. This hydrophobic drug analog exhibits a 4-log increase in antiviral activity against herpesvirus over cidofovir (**Figure 2**).¹⁸

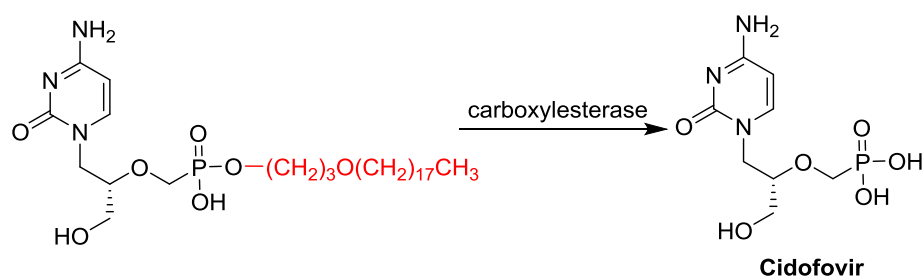


Figure 2. Enzymatic hydrolysis of the prodrug octadecyloxypropyl cidofovir, which gives rise to the drug cidofovir inside the cell.

Cell penetrating peptides (CPPs): These are usually short, positively charged peptides (generally less than 30 residues) that can translocate across cell membranes at low micromolar concentration without harming the cellular integrity. The first known CPP was discovered from a viral protein possessing a peptidic segment that enables the entire protein to penetrate the cellular membrane.¹⁹

After this finding, an entire research field emerged, which has led to the discovery of a large variety of natural, synthetic, and non-peptidic CPPs. In fact, more than 800 different CPPs have been reported so far.²⁰ Although the uptake mechanism of CPPs is complex, they are believed to adsorb onto the negatively charged cell membrane due to electrostatic interactions, which induce their uptake by several endocytic pathways: caveolae-mediated, clathrin-mediated, and macropinocytosis, followed by endosomal escape.²¹ CPPs can be covalently bound to small molecules to improve their cellular uptake, and even to allow them crossing biological barriers such as the blood-brain-barrier. Using small drugs such as benzylpenicillin and doxorubicin, it has been proven that the amount of drug transported into the brain can be enhanced up to 20-fold when the drug is covalently attached to a CPP.²² It should be noted that both entities should be linked such that the drug activity is not affected, which is probably the main limitation of combining CPPs with small molecules.

A new class of promising CPPs are activatable cell penetrating peptides (ACPPs). They consist of a polycationic CPP bound to an inhibitory polyanion, and connected through a cleavable linker.²³ The polyanion neutralizes the polycation and largely inhibits the CPP's cell adhesiveness. Upon cleavage of the linker, the CPP is released and internalized into nearby cells. Through appropriate linker design, ACPPs have been directed toward extracellular enzymes, thereby concentrating the cargo of interest at the site of activation, where the enzyme is present. On the other hand, ACPPs can also be designed to respond to external stimuli, and in this way the spatio/temporal control of the cell uptake of molecules can be achieved.²⁴ For instance, a new kind of ACPPs has been recently described, whose uptake can be induced by a host-guest complex, involving an anion recognition process (**Figure 3**).²⁵ This approach relies on the encapsulation of a negatively charged pyranine that quenches the cellular uptake of a CPP. Using an oligocationic

covalent cage,²⁶ the negatively charged pyranine can be transformed into a positively charged host-guest complex so that the quenching effect is relieved, and the ACPD activated. Interestingly, none of the components, the cage or the pyranine-peptide guest, are able to cross the cell membrane as separate units, but their association promotes the efficient uptake of both components simultaneously.

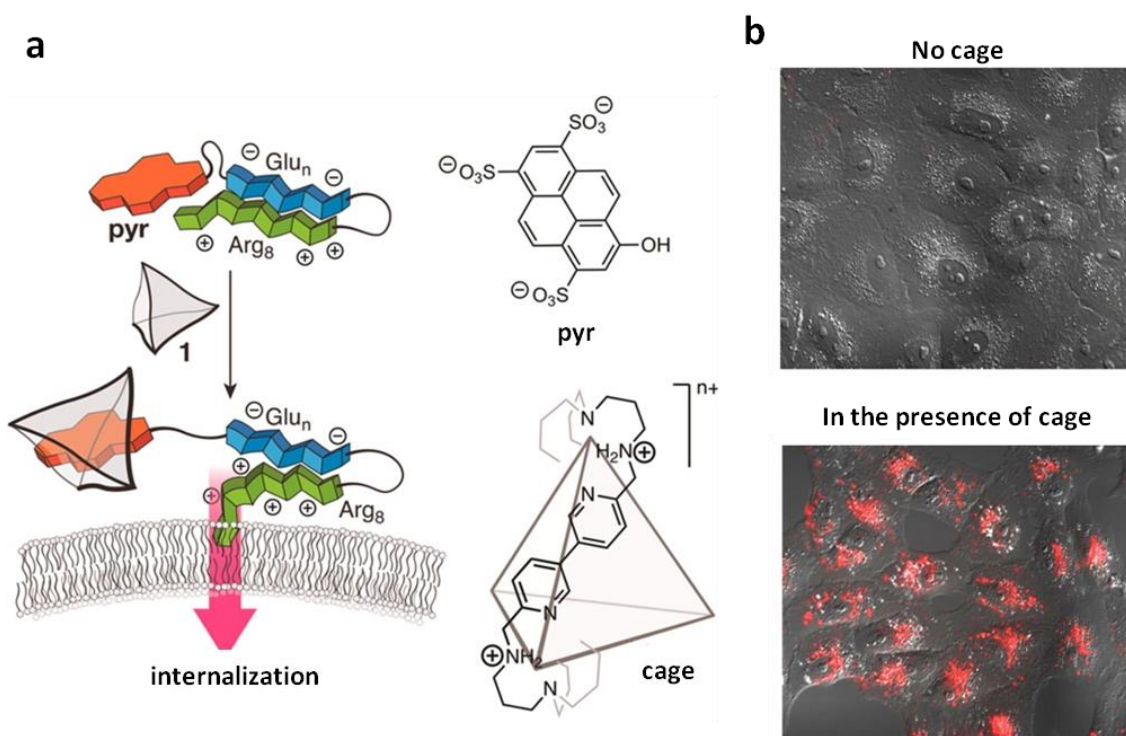


Figure 3. a) An activable cell penetrating peptide based on a CPP (Arg₈) electrostatically masked by a polyanionic pyranine-Glu₆ domain that can be activated by the formation of a supramolecular host-guest complex between pyranine and the positively charged cage. b) Fluorescence micrographs of Vero cells after incubating the ACPD bound to a rhodamine dye, in the absence (top) or the presence (bottom) of 1 equiv of cage. Reproduced with permission from ref. ²⁵. Copyright 2017 ACS.

CELLULAR UPTAKE OF BIOLOGICAL MACROMOLECULES

Proteins and nucleic acids are promising therapeutic agents for the treatment of various diseases.²⁷ In most cases, cell internalization of these large molecules are indispensable for them to accomplish their biological functions. Unfortunately, the cellular uptake of proteins and nucleic acids is very inefficient for different reasons. First of all, due to their size (>1 KDa), macromolecules are unable to cross the cell membrane by simple diffusion. Second, endocytic pathways have low efficiency in the concentration ranges that these polymers are typically used, with the exception of those that interact with a specific receptor. Third, after endocytosis macromolecules remain trapped in endosomes, from where they are delivered to lysosomes to be hydrolyzed.²⁸ Therefore, in contrast to small molecules for which a simple rule can predict internalization by simple diffusion and direct access to the cell cytoplasm; macromolecules are in general poorly taken up by cells, and end up being hydrolyzed inside lysosomes. Interestingly, covalent attachment of fluorescent dyes to these large molecules does not significantly influence their cell internalization, thus allowing straightforward quantification of the uptake.

Approaches to improve cell uptake of biological macromolecules

Cell penetrating peptides: As in the case of small molecules, CPPs can also be used to deliver macromolecules into the cellular cytoplasm.²⁹ However, there are some difficulties associated to the larger size, as it has been reported that cargos like proteins and DNA can alter the internalization mechanism and endosomal escape efficiency of CPPs.³⁰ A clear example of this different behavior is the case of a nonpeptidic CPP based on guanidinium units. When this CPP is bound to small molecules such as fluorescent dyes, it specifically accumulates in mitochondria,³¹

but when the same CPP is attached to a peptidic fragment of the GCN4 protein (24 amino acids), the resulting conjugate is completely trapped in endocytic vesicles.³²

Despite the difficulty of using CPPs with large molecules, an important advantage is related to the larger size. CPPs are usually positively charged molecules, in some cases also hydrophobic. When the cargo is negatively charged or hydrophobic, it is possible to achieve non-covalent complexation with CPPs, due to a large number of cooperative interactions between both molecules.³³ The main advantage of this strategy lies in the fact that the intermolecular interactions are reversible, so the biological activity of the cargo is not compromised by covalent connection with the CPP. This non-covalent strategy has been extensively used for the cellular delivery of nucleic acids.³⁴

As mentioned above, one of the main problems of using CPPs is that most cargos remain entrapped in endosomes, and cannot reach their cytosolic targets. The most widely applied strategies to reduce endosome trapping is the use of endosomolytic peptides and membrane-disruptive polymers, bound to CPPs.³⁰ Endosomolytic peptides are able to break endosome membranes, but have a low tendency to damage the cell membrane because the lysis activity is triggered by endosome acidification. On the other hand, membrane-disruptive polymers can also rupture the endosome upon acidification, due to a proton sponge effect.³⁵

Interestingly, a new class of short peptide has been recently reported, which acts simultaneously as CPP and endosomolytic peptide.³⁶ This peptide derives from the membrane-lytic spider venom peptide M-lycotoxin, in which one amino acid from the hydrophobic part of the natural peptide was replaced by a negatively charged glutamate. The mutated peptide is still able to adsorb onto the cell membrane due to its highly positive charge, thus it is quickly endocytosed, but its lytic

activity is dramatically reduced. Upon endosome acidification, the glutamate amino acid is protonated and the membrane-lytic activity recovered, thereby releasing the endosome contents into the cytosol (**Figure 4**). This CPP has three important advantages: i) it stimulates physiological uptake of the cargo via the induction of macropinocytosis, ii) it is compatible with non-covalent approaches, and iii) it is able to break endosomes and release their cargo into the cytosol.

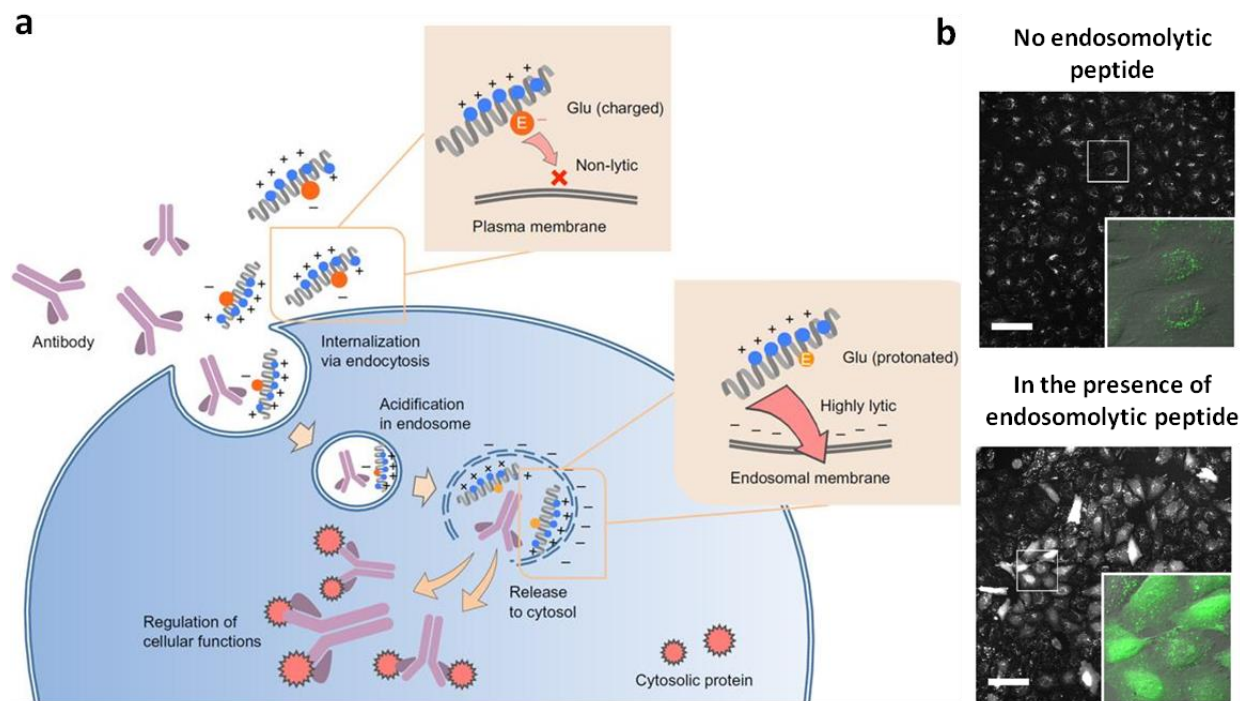


Figure 4. a) Schematic illustration of the cellular internalization mechanism for a short peptide derived from M-lycotoxin, which is able to release macromolecules such as antibodies into the cytosol. Upon endosome acidification the peptide recovers its lytic activity and releases an antibody from the endosome. b) Fluorescence microscopy images of HeLa cells incubated with Alexa488–dextran (10 kDa), in the absence and in the presence of the endosomolytic peptide. Scale bars: 100 μ m. Reproduced with permission from ref. ³⁶. Copyright 2017 Springer Nature..

Strain-promoted thiol: Reactive disulfides can enter cells by covalent attachment through a disulfide exchange with cellular external thiols.³⁷ This is the case of highly strained cyclic disulfides, which react mainly with the transferrin receptor (TFRC). Once cyclic disulfides are covalently bound to the receptor, they are transported across the cellular membrane (**Figure 5**). Importantly, the uptake efficiency of these analogs increases with the disulfide ring tension. This strategy has been successfully applied to the cytosolic delivery of both small and large molecules.³⁸

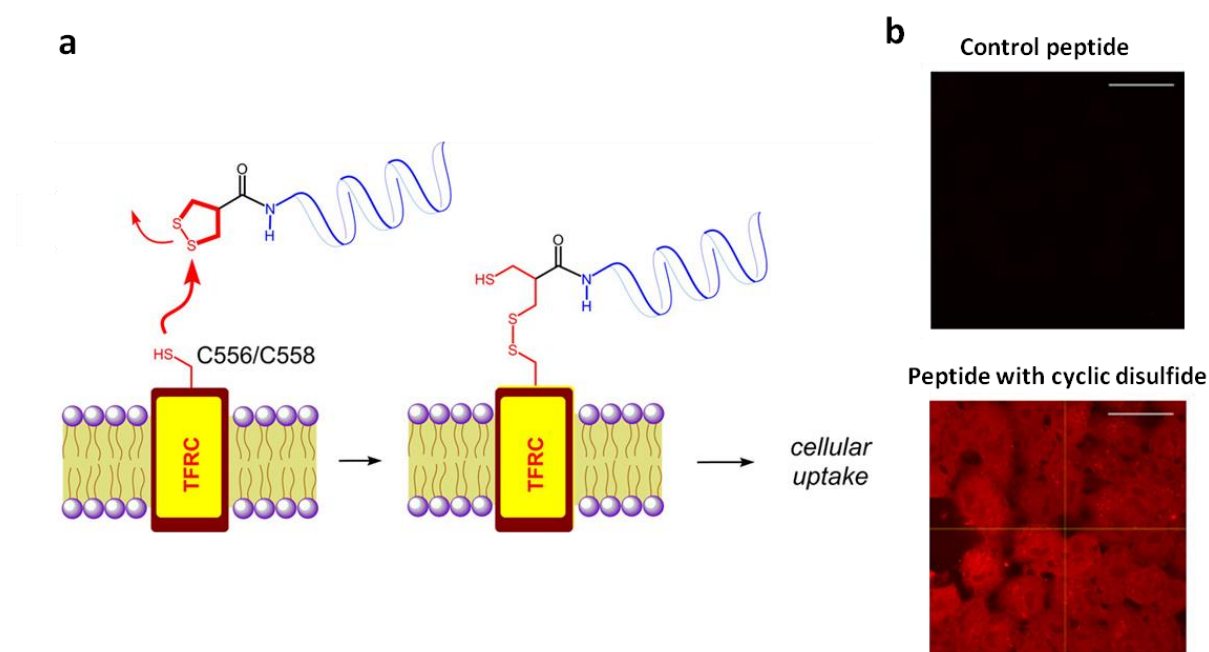


Figure 5. a) Schematic representation of the strain-promoted thiol uptake, applied to cell internalization of peptides. Solvent-exposed cysteines on the surface of TFRC react with the strained disulfide ring, and then the peptide is taken up by the cell. b) Fluorescence microscopy images of HeLa cells incubated with a peptide, without (control) and with a strained cyclic disulfide. Scale bars, 30 μm . Reproduced with permission from ref. ³⁷. Copyright 2017 ACS.

Synthetic polymers: Polymers have been largely used to achieve cellular uptake of nucleic acids, which are essential components of gene therapy.³⁹ Positively charged polymers form

supramolecular assemblies with DNA and RNA, through electrostatic interaction, to generate polyion pairs. These polyions bind onto the cell surface by non-specific, electrostatic interactions between the positively charged complexes and the negatively charged cell surface, entering cells via endocytic mechanisms. Once inside the cell, the endosomal pH drops from 7 to 5.5 and the endosome is disrupted by the proton sponge effect. Some examples of these polymers are polyethylenimine and polyamidoamine dendrimers (PAMAM).⁴⁰

CELLULAR UPTAKE OF NANOPARTICLES

Nanoparticles (NPs) are exogenous synthetic structures with nanoscale dimensions, which have raised enormous interest toward biological applications. Owing to their comparatively large size, the cellular uptake of NPs is necessarily different to that of molecules. For example, while most molecules are unable to internalize cells efficiently on their own (*vide supra*), NPs are actively incorporated into the cell via different endocytic pathways.⁴¹ Additionally, owing to their efficient uptake, engineered NPs have been proposed to improve the cellular permeability of small molecules and proteins.⁴² The reason behind this uptake capability is that NPs feature a large and highly energetic surface, which is available to interact with biomolecules, such as those forming the cell membrane. An extensive network of cooperative weak interactions result in a high affinity between NPs and cells. However, this effect also has some drawbacks; when NPs are dispersed in biological fluids, diverse types of biomolecules (mainly proteins) readily adsorb onto the NPs forming the so-called protein corona (PC), which may dramatically change their biological identity, thereby affecting the uptake process and potentially blocking their targeting capabilities.⁴³

Approaches to improve cell uptake of NPs

Modulation of NP physicochemical properties: Although endocytic uptake is the norm for a broad range of NPs, various structural features should be considered, including size, shape and surface chemistry.⁴¹ Although there is no consensus regarding an optimum size that maximizes the level of cellular uptake, it seems clear that e.g. 50 nm Au NPs are taken up by cancer cells (HeLa) in larger amounts than both smaller and larger Au NPs, which require specific functionalization to achieve a similarly efficient delivery into cells.⁴⁴ Surface charge is another property that plays a major role in the interaction with cells, so that positively charged NPs are often more efficiently internalized than negatively charged and neutral NPs of similar dimensions (**Figure 6**).⁴⁵

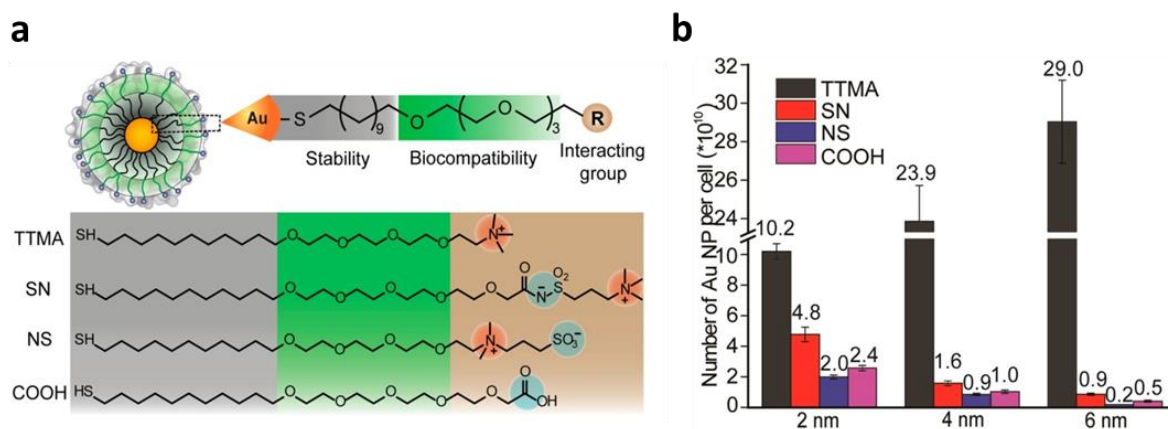


Figure 6. a) General structure of 2 nm Au NPs, with either cationic, anionic or zwitterionic headgroups. b) Cellular uptake of as-synthesized gold nanoparticles with different sizes and surface charge, by HeLa cells after 3 h incubation in serum-free media. Reproduced with permission from ref. 46. Copyright 2015 ACS.

An important challenge in this field comprises the development of strategies toward regulating cell internalization by means of chemical or physical stimuli. On the basis of the surface charge effect, two approaches have been described to control the cellular uptake of small Au NPs (2 nm).

The first one involves coating the NPs with organic ligands that are neutral at pH 7.4, but become positively charged at acidic pH (< 6.5), thus increasing the cellular uptake in acidic environments, which are typical of tumor tissues.⁴⁶ The second is a supramolecular approach based on the same host-guest strategy described in **Figure 3** above, where NPs decorated with pyranines are unable to cross cell membranes owing to their high negative surface potential. However, they are efficiently internalized upon addition of the oligocationic cage, which interacts with pyranine, forming a positively charged host-guest complex and thereby reversing the overall surface charge. In contrast with the pH-responsive strategy, it has been proven that the latter method can be used to reversibly turn on/off NP internalization,⁴⁷ thereby allowing a precise spatio/temporal control.

Active targeting systems: A number of active delivery strategies have been tested, through attachment of high affinity ligands onto the surface of NPs. Internalization can then occur via receptor-mediated endocytosis. A broad type of ligands conjugated to metallic NPs have been used for such purposes, such as small molecules, carbohydrates, aptamers, or proteins.⁴⁸ It is important to optimize the density of targeting ligands per NP to achieve high degrees of targeting efficiency and internalization. For instance, Au NPs with varying densities of cetuximab antibodies, which are active against the epidermal growth factor receptor, exhibit different uptake mechanisms.⁴⁹ Interestingly, the use of actively targeted NPs can enhance drug retention, minimize nonspecific uptake, and circumvent drug degradation mechanisms.

Protein corona modulation: Adsorbed proteins confer a different biological identity to NPs, which may completely alter the subsequent cellular responses.⁵⁰ Biomolecules can be bound through irreversible interactions, forming the so-called *hard corona*, or through weak interactions, yielding a *soft corona*. The NPs-protein complex is a complicated and dynamic entity, and its composition may change with time and environmental alterations, through continuous protein association and

dissociation.⁵¹ PC usually reduces the direct contact of NPs with cell membrane components, producing an inhibition of the cellular uptake of NPs. However, this effect also depends on NP size, protein corona composition and cell type.

The PC composition strongly influences NP interactions with cells. For example Apolipoprotein A mediates Au NP cell association, which has been used for brain targeting strategies, whereas the presence of Immunoglobulin G in the PC can inhibit this effect.⁵² PC formation may reduce or even block the targeting capabilities of NPs, by inhibiting the binding with their target site. Coating of NPs with polyethylene glycol (PEG) has proven useful to partly inhibit PC formation, and this effect has been used e.g. to restore the targeting ability of Herceptin-targeted AuNPs.⁵³ An alternative protective coating is provided by low-molecular-weight glycans, which have been used to stabilize Au NPs of different shapes in biofluids. Glycan-coated NPs were resistant to adsorption of proteins from serum-containing media and to prevent phagocytosis by macrophage-like cells, but retained carbohydrate-binding targeting capabilities toward tumor cells (**Figure 7**).⁵⁴

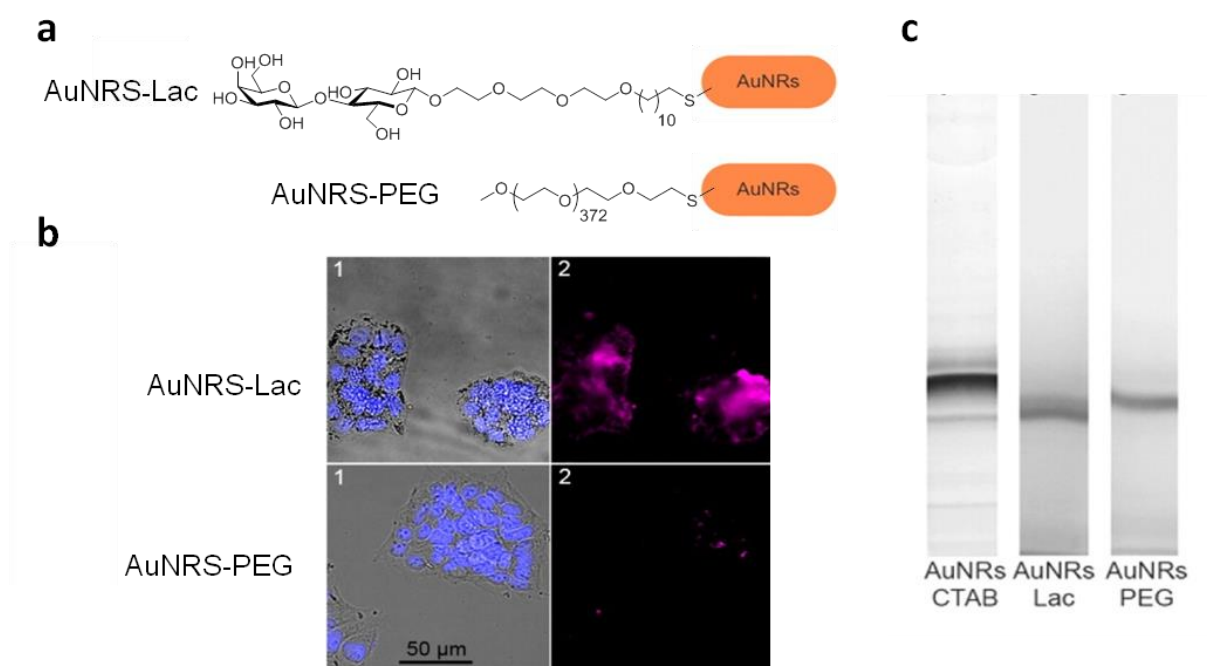


Figure 7. Biological behavior of gold nanorods stabilized with long PEG ligands and with a short organic ligand containing lactose (Lac). a) Organic structure of the ligands. b) Fluorescence microscopy images of DLD1 cancer cells incubated with fluorescently labeled AuNRs-Lac (upper panel) and AuNRs-PEG (lower panel). (1) Brightfield and DAPI fluorescence overlay. (2) Fluorescence signal from AuNRs. c) Comparison of the degree of protein immobilization on AuNRs coated with different ligands, upon exposure to 10% *fetal bovine serum* in phosphate-buffered saline. CTAB is hexadecyltrimethylammonium bromide. Reproduced with permission from ref. ⁵⁴. Copyright 2015 ACS.

Endosomal escape: Many biomedical applications will ultimately require intracellular delivery, as well as availability of the NP, not only to a certain type of cells, but also to specific subcellular compartments. Although emphasis is often placed on the importance of regulating NPs uptake, endosomal escape is perhaps the most challenging barrier against the delivery of NPs. Although several approaches have been explored to achieve the release of NPs from endosomes, no strategy has been proven efficient so far, without damaging the cells. We highlight here three strategies; binding of polymers that can disrupt the endosome by the proton sponge effect,⁵⁵ nanocapsules that can internalize the cell through membrane fusion for internalization of 2 nm NPs,⁵⁶ and laser irradiation of plasmonic Au NPs.⁵⁷

CONCLUSIONS AND OUTLOOK

We have shown that size is an important parameter that must be considered when aiming at an efficient cellular uptake for a synthetic structure. Each range of sizes has their own advantages and disadvantages. In summary, small and lipophilic molecules can cross the cell membrane by simple diffusion and access all cell compartments, whereas internalization of small polar molecules and

macromolecules is challenging. On the other hand, NPs are easily taken up by cells, but they usually remain trapped in endosomes. Although we present in this Account several strategies that can be used to improve the cell internalization properties for each size range, there is still a long way to go before cellular uptake is mastered. Particularly challenging is avoiding endosomal entrapment of NPs and macromolecules. Although several strategies have been developed to solve this problem, only a small part of the internalized chemical structures are usually able to escape lysosomal degradation, using currently existing methods.

In contrast, medicine is currently entering the age of precision and personalized treatments; it seems thus likely that future medicine will be mainly based on the use of proteins and nucleic acids as future drugs, while NPs may act as drug delivery platforms that are selective toward a specific kind of cells. Therefore, there is a great need for strategies that can bypass lysosomes, so that large drug carriers can reach the inner side of cells. A possible solution is the development of chemical vectors that induce selectively cell internalization by caveolin-mediated endocytosis, which is used by some viruses and bacteria, to avoid lysosomal degradation.⁵⁸

On the other hand, an ideal drug delivery platform must be able to internalize only the target cells, and to subsequently release the drug avoiding side effects. In order to achieve this goal, efficient ways to reduce or modulate PC formation on NPs must be identified. The performance of the current methods, mainly based on PEG ligands as coating agents, are far from ideal. As a consequence, the physico-chemical properties of NPs change in the presence of biological media, thereby hindering their targeting capabilities.

Finally, it is also worth noting that a major challenge in this area comprises the development of approaches to achieve spatio/temporal control of cellular internalization using external stimuli. We discussed above several ways to activate cellular uptake based on chemical stimuli, but such

strategies are difficult to apply in living organisms. Additional research should be carried out to achieve the same performance but applying physical stimuli such as near infrared light, magnetic fields or ultrasound, which are harmless to biological tissues.

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